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Zinc Chloride Activates Phospho-Akt and Promotes Chondrocyte Maturation in the ATDC5 Chondrogenic Cell Line

Donya T. Burgess
donya.burgess@student.shu.edu

Zinc Chloride Activates Phospho-Akt And Promotes Chondrocyte Maturation In The ATDC5 Chondrogenic Cell Line

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Donya T. Burgess

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Dr. Jessica Cottrell, PhD

Mentor

Dr. Daniel Brian Nichols, PhD

Committee Member

Dr. Allan Blake, PhD

Committee Member

Dr. Angela Klaus, PhD

Director of Graduate Studies

~hD

Chair of Biological Sciences

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Abstract

Fracture healing is characterized by inflammation, cartilage formation, bone deposition, and remodeling. Endochondral ossification is an important part of callus formation that relies on the maturation of chondrocytes. Early in their maturation these cells express collagen type II alpha 1 chain (COL2A1) and express collagen type X alpha 1 chain (COL10A1) as they mature further and become hypertrophic chondrocytes. Activation of the insulin pathway is considered a primary factor involved in the initiation of chondrogenesis and its progression during bone healing. Insulin mimetics like zinc chloride ($ZnCl_2$) have been shown to improve bone healing outcomes and enhance bone healing, but the mechanism by which insulin mimetics enhance bone healing is unclear. We hypothesized that treatment of chondrocytes with $ZnCl_2$ would activate AKT within the insulin-signaling pathway and promote chondrocyte maturation and differentiation similar to insulin. The ATDC5 chondrogenic cell line was treated zinc chloride ($ZnCl_2$) and evaluated for chondrogenesis by measuring calcium deposition, proteoglycan synthesis, protein expression, and gene expression at specific timepoints between 0-28 days. Our data demonstrates that $ZnCl_2$ induces the insulin pathway by phosphorylation of Akt. $ZnCl_2$ treatment promotes chondrogenesis as seen by increased expression of chondrogenic markers COL2A1 and COL10A1 and calcium deposition.

Keywords: chondrocyte, maturation, zinc, insulin, diabetes, fracture, regeneration, ossification

Introduction

Diabetes and Bone Healing

Diabetes mellitus is a metabolic disorder which is known to have improperly regulated insulin levels. Many complications associated with diabetes exist including decreased bone mineral density and impaired bone healing [6]. The bone impairment is projected to rise from 171 million patients in 2000 to 366 million in 2030 [26]. Research has shown that insulin given locally at fracture sites accelerates fracture healing in both diabetics and non-diabetics. Insulin has been shown to be essential to the upregulation of chondrogenesis. Insulin insufficiency, hyperglycemia and oxidative stress are hallmarks of both Type 1 diabetes (T1DM) and Type 2 diabetes (T2DM). These diabetic conditions reduce osteoblast differentiation, increase osteoclast activity, and alter apoptosis of chondrocytes and osteoblasts leading to impaired fracture healing in diabetic patients [14-21]. Clinical studies also show that diabetes delays fracture healing. In diabetic patients, fracture union time (normal fracture healing) may be prolonged by 87%. Diabetes is also associated with a relative risk of 3.4-fold for complications in osseous healing, including delayed reunion (prolonged fracture healing), non-union, redislocation or pseudoarthrosis [10]. Diabetics have slower healing rates and have higher risk of re-injury than non-diabetics. Local insulin treatment applied at the fracture site in both diabetics and non-diabetics, demonstrated accelerated fracture healing [10]. Topping et al [32] reported a 54-70% decrease in Type X collagen in the fracture callus of diabetic rates and suggested that this might have a role in defective diabetic fracture healing. These studies support the theory that diabetes might affect fracture healing at the stage of cartilage formation [10, 32].

Bone regeneration is a complex, well-orchestrated physiological process of bone formation, which can be seen during normal fracture healing, and is involved in continuous

remodeling throughout adult life [1]. Fracture healing is a common form of bone regeneration and is characterized by inflammation, cartilage formation, cartilage bridging, bone deposition, and remodeling. The ability of fractures to regenerate and undergo repair may be compromised when an insufficient osteogenic reaction is observed in the fracture callus, and can result in atrophic nonunion [30]. An atrophic nonunion occurs when no callus is formed often due to impaired bone healing caused by vascular (e.g. impaired blood supply) or metabolic complications (e.g. diabetes). These atrophic nonunions are solved with bone healing augmentation instead of a mechanical approach which would employ the use of nails, pins, or other hardware. Immediately following a fracture, a hematoma forms at the site of injury (primary fracture healing) acting as a protective blood clot to mediate the inflammatory process and contribute to the removal and replacement of necrotic bone with a new bone matrix (secondary fracture healing) [4]. The hematoma contains fracture debris and initiates a pro-inflammatory cascade by recruiting immune cells from the surrounding soft tissues, lymphatic system, and vasculature. Within a hematoma a low pH microenvironment is formed where inflammatory cells secrete pro-inflammatory cytokines [tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and IL-6] to activate the polymorphonuclear neutrophils and M1 (or pro-inflammatory) macrophages that are recruited to manage this acute inflammation stage [40]. Traumatic inflammation during fracture healing is a key sign that inflammatory cells and bone cells are interacting with each other through common cytokines, receptors, transcription factors, and signaling molecules due to communication between cells of the monocyte-macrophage-osteoclast and MSC-osteoblast lineages [8]. Fracture healing benefits from an inflammatory response triggered by the immune system to stimulate secretion of signaling molecules that contribute to the healing process. For example, matrix metalloproteinases (MMPs) are a family

of zinc-dependent endopeptidases that play a crucial role in various physiological processes including tissue remodeling and organ development, in the regulation of inflammatory processes, and in diseases such as cancer [92]. Inflammatory cells immediately collaborate with the complement system, coagulation cascade, and neutrophils along with “emergency signals” triggered by cytokine/chemokine network post-trauma as the “first line of defense” initiated by innate immunity.

Indirect (secondary) fracture healing has other key events that proceed hematoma formation, such as acute inflammation (neutrophils and macrophages are recruited, and pro-inflammatory cytokines are produced), granulation tissue formation (angiogenesis), callus formation (MSCs differentiated into chondrocytes) and remodeling (formation of chondroblasts and osteoclasts) [5]. Inflammatory cytokines are known to have negative effects on bone, joints, and implanted material when prolonged or chronic expression occur [34]. Tissue regeneration requires a brief yet highly critical regulated secretion of inflammatory cytokines immediately following an acute injury. Acute inflammatory response peaks within the first 24 hours and is complete after 7 days [35]. Secretion of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), MMPs, interleukin-1 (IL-1), IL-6, IL-11, and IL-18 are known to promote angiogenesis and recruit other proinflammatory cytokines initially an acute injury (Figure 1). MMPs participate in regulating mechanisms in all of repair processes. MMPs can activate cytokines and chemokines by cleaving them from the cell surface or processing them to increase their activity, or degrade them, thereby inhibiting inflammatory signals. MMPs are involved in remodeling the ECM either directly by proteolytic degradation of proteins, such as collagens, or indirectly via their ability to affect cell behavior. Alteration of the ECM is integral to the resolution of wound healing but also has implication in regulation of inflammation [92].

Figure 1: An overview of cytokine-mediated regulation of synovial interactions. *Source McInnes & Schett (2007).*

The TNF-α concentration has been shown to peak at 24 hours and return to baseline within 72 hours post trauma [18]. Within that timeframe, TNF-α is expressed by macrophages, and act as a chemotactic agent to recruit necessary cells such as neutrophils, chondrocytes, and osteoblasts [36]. *In vitro* studies have shown that TNF-α induces osteogenic differentiation of MSCs [36-38]. Osteogenic differentiation is mediated by TNFR1 and TNFR2 receptors expressed on both osteoblasts and osteoclasts. Interleukins, IL-1 and IL-6, are expressed during osteogenic differentiation and is believed to be most important for fracture healing [35-38]. IL-1 is produced by macrophages in the acute phase of inflammation and induces production of IL-6 in osteoblasts, promotes the production of the primary cartilaginous callus, and promotes angiogenesis at the injured site by activating either of its two receptors, IL-1RI or IL-1RII [36-38]. IL-6 is only produced during the acute phase and stimulates angiogenesis, vascular endothelial growth factor (VEGF) production, and the differentiation of osteoblasts and osteoclasts [39]. The anti-inflammatory state is due largely to the phenotypic modulation (transition from M1) of macrophages to the M2 (or alternatively activated, anti-inflammatory

macrophages) population in the fracture callus. These anti-inflammatory M2 macrophages secrete a battery of cytokines and growth factors to promote tissue repair and angiogenesis, such as, IL-10, PDGF, VEGF, transforming growth factor-β (TGFβ), epidermal growth factor (EGF), and arginase [40].

Figure 2: Femur fracture repair. *Source Einhorn, T. A., & Gerstenfield, L.C. (2014).*

Angiogenesis is the formation of new blood vessels which is controlled by chemical signals that can stimulate both repair of damaged blood vessels and formation of new blood vessels [42]. Angiogenesis involves migration, growth, and differentiation of vascular endothelial cells aligned inside the wall of blood vessels [42]. Local and systemic factors such as fibroblast growth factor (FGF), transforming growth factor (TGF), bone morphogenetic protein (BMP) and vascular endothelial growth factor (VEGF), are active during fracture healing (Figure 2) and are known to have direct or indirect chondrogenic, osteogenic and/or angiogenic functions. Endogenous VEGF is a key player in bone repair, where its temporal and spatial expression pattern corresponds to the one observed during long bone development [42, 43]. VEGF is a critical growth factor for normal angiogenesis and appropriate callus architecture and

is induced in osteoblasts. It regulates recruitment, survival and activity of osteoclasts, endothelial cells, and osteoblasts [43]. Also, VEGF has been observed to play a major role in cartilage maturation and resorption. Produced by hypertrophic chondrocytes, VEGF initiates the endochondral ossification cascade by recruiting and/or differentiating osteoclastic cells that resorb cartilage and by attracting osteoblasts [44, 45]. Inhibition of VEGF activity disrupts angiogenesis and results in non-unions (i.e. a failure to establish a bony bridging of the fracture gap) and delayed unions (i.e. a failure to establish a bony bridging of the fracture gap within a clinically reasonable period of time approximately 3-12 weeks for common fractures) [43]. Therefore, uninhibited growth factor activity is required to promote angiogenesis and appropriate bone bridging formation during fracture healing.

Bone Formation

There are two major modes of bone formation that involve the transformation of a preexisting mesenchymal tissue into bone tissue: intramembranous ossification and endochondral ossification [2]. Intramembranous ossification is the process of direct bone formation, where osteochondral progenitors differentiate directly into osteoblasts [40]. Intramembranous ossification also occurs in the bones of the skull where neural crest-derived mesenchymal cells proliferate and condense into compact nodules [2]. Osteoblasts synthesize an ECM containing Type I collagen. During intramembranous healing, bone spicules created from the osteoblasts fuse to form a trabeculated bone that is eventually fully remodeled into a cortical bone ultrastructure through the coordinated action of osteoblasts and osteoclasts [40]. Endochondral ossification is an indirect bone formation process and an important mechanism for callus formation that relies on the differentiation of mesenchymal stem cells (MSCs). Cartilage is produced via maturation of chondrocytes and is later replaced by bone.

All bones from the base of the skull down, except for the clavicles, are formed this way. The process of endochondral ossification can be divided into five stages. First, MSCs committed to endochondral ossification differentiate into chondroblasts. Chondroblasts secrete cartilage ECM, resulting in a cartilage model of the bone. Second, committed MSCs condense into compact nodules and differentiate into chondrocytes that proliferate rapidly to form the model for the bone (cartilage-specific extracellular matrix (ECM). The cartilage model continues to grow as chondrocytes divide and more ECM is secreted, a processc called interstitial growth. Appositional growth ensues when new chondroblasts in the perichondrium secrete matrix on the periphery of the cartilage model. Chondrocytes stop dividing when ECM is formed and become hypertrophic deep in the center of the cartilage model. Hypertrophic chondrocytes stop secreting collagen and begin to secrete an enzyme essential for mineral deposition called alkaline phosphatase that then cause the ECM to calcify. Calcification causes remaining chondrocytes to die because nutrients are difficult to transfer through a calcified matrix. Third, invasion of cartilage ECM model by expanding blood vessels and terminally differentiated hypertrophic chondrocytes die by apoptosis or undergo osteogenic transdifferentiation [2]. Osteoblasts begin to secrete bone matrix around any remaining calcified cartilage and begin form the primary ossification center. Fourth, primary ossification center spreads as osteoclasts break down newly formed bone. This causes formation of the medullary cavity in the diaphysis; walls of the diaphysis will eventually be replaced by compact bone. Last, secondary ossification centers form in the epiphyses following invasion by branches of the epiphyseal artery.

Fracture repair initially begins with the formation of fracture hematoma at 6-8 hours post injury. Within the fracture line, blood vessels are broken causing blood to leak out and form a significant clot around the fracture site. Due to lack of blood circulation near fracture site, nearby

bone cells die, and inflammation occurs at the affected area. Fibrocartilage callus formation takes about 3 weeks to occur. Fibroblasts from the periosteum migrate to the fracture site and produce collagen fibers. Some cells differentiate into chondroblasts to produce fibrocartilage. Fibrocartilage callus is composed of collagen fibers and cartilage that bridges the gap in injured tissue. At approximately 3-4 months, bony callus forms in areas close to healthy tissue. Osteogenic cells differentiate into osteoblasts which begin to lay down spongy bone. Later, spongy bone tissue replaces fibrocartilage callus. Lastly, osteoclasts resorb dead pieces of broken bone and spongy bone is replaced by compact bone along the periphery of the fracture (bone remodeling).

Chondrocyte Maturation

Chondrogenic differentiation of bone marrow-derived stromal cells (BMSCs) is a complex interactive network between transcriptional factors, extracellular growth factors, and signal transduction pathways [47,48] (Figure 4). Several comparative studies have shown that the quality of cartilage produced by using BMSCs is substantially lower than that obtained by using chondrocytes [46]. Chondrocytes are cartilage-producing cells, which are derived from mesenchymal stem cells. The early steps of chondrogenesis, including mesenchymal condensation and the expression of chondrocyte-specific extracellular proteins, are crucially dependent upon SRY-related HMG box (Sox) family transcription factors, including Sox9, Sox5 and Sox6 [51] (Figure 3).

By contrast, Runt-related transcription factor-2 (RUNX2) regulates the process of chondrocyte hypertrophy and Runt-related transcription factor 3 (RUNX3) and Forkhead box A2/3 (Foxa2/3) [51]. Intrinsic chondrogenic differentiation potential of BMSCs is believed to be

controlled by transcription factors Sox-9 and RUNX-2, whereas TGF, like TGF-β3, as well as BMPs are some of the most potent inducers of BMSC chondrogenesis [46] (Figure 4). Early in their maturation chondrocytes express collagen Type II alpha 1 chain (COL2A1). During further

Figure 3: Different combinations of transcription factors drive tissue-specific gene expression in immature versus hypertrophic chondrocytes. *Source Kozhemyakin et al. (2015)*

maturation, chondrocytes express crucial gene marker such as collagen Type X alpha 1 chain (COL10A1). The stromal cell-derived factor-1 (SDF1)/chemokine receptor (CXCR) pathway is a key regulator for BMSC migration, and, in the absence of SDF1 signal, migration of these cells to the bone tissue has been found to be impaired [49, 50]. The process of chondrogenic differentiation includes six phases: mesenchymal cells (chondroprogenitors), condensed mesenchymal cells, chondrocytes, proliferating chondrocytes, pre-hypertrophic chondrocytes, and hypertrophic chondrocytes [3]. Endochondral ossification, therefore, requires chondrogenesis and cartilage hypertrophy as necessary precursors before ossification can proceed.

Figure 4: Possible mechanisms operative in cartilage regeneration by mesenchymal stem cells. *Source Gupta et al. (2012)*

Activation of Insulin Pathway

Activation of the insulin pathway is considered a primary factor involved in the initiation of chondrogenesis and its progression during bone healing [9]. Insulin is a polypeptide hormone that directly and indirectly modulates bone metabolism and skeletal regeneration among other physiological events. The insulin receptor (IR) belongs to a family of receptor tyrosine kinase (RTK) transmembrane signaling proteins, which include insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors [56]. The IR is initially synthesized as a proreceptor and cleaved to form a 350-400kDa heterotetrameric receptor composed of two α-subunits and two β-subunits joined by disulphide bones in a β-α-α-β configuration as illustrated in Fig. 5 [57]. During receptor activation, insulin binds to one of two binding sites on each of the αβ monomers called, site 1 and site 2 [58]. When insulin first binds to site 1, a high-affinity cross-linking reaction takes place, allowing site 2 to

become accessible for ligand binding to activate the receptor [59, 60]. Once bound, the α-subunit which normally prevents autophosphorylation of the β-subunit, is inhibited causing the kinase

Figure 5: Insulin receptor and the major signal transduction pathways used in tissue repair, biomacromolecules synthesis and glucose uptake. *Source Hrynyk et al. (2014)*

activity of the β-subunit to transphosphorylate, changes conformation and increases its own kinase activity [61]. The signaling cascade begins with the phosphorylation of intracellular tyrosines located on the β-subunits of the receptor. The phosphorylated tyrosines act as docking sites, permitting the Shc-transforming protein (Shc) found in the cytosol to bind via their Src-homology-2 (SH2) domains [62-64]. The signal is then transduced via a series of messenger molecules including Grb2 (growth factor receptor-bound protein-2), which recruits SOS (son of sevenless), to activate Ras [65]. After activation, Ras (member of the GTP-binding protein family) carries the signal via mitogen-activated protein kinase kinase kinase (Raf or MAP3K), mitogen-activated protein kinase (MEK) and extracellular signal-related (ERK) protein kinases. ERK then translocates into the nucleus where cell transcription is achieved, cell cycle activation occurs and DNA replicate [62]. The IR signaling pathway leads to tissue repair and wound healing.

Several studies have indicated that local insulin treatment may potentially enhance osseous or fracture healing in non-diabetic animals [52]. In non-diabetic mice, injection of insulin over the calvarium increased osteoid area, osteoblast surface area, and osteoblast number [54]. Dedania et al. found that local insulin delivery significantly increased the percent of mineralized tissue within the endosteal region of the healing defect at 4 and 6 weeks post-osteotomy, compared with saline control animals [55]. Functional insulin receptors on rat osteoblasts have been identified that, when activated, stimulate osteoblast proliferation, and increase collagen production *in vitro* [53]. Insulin has been postulated to elicit an anabolic role in bone via insulin receptor (INSR); however, recent data has shown that the anabolic effect on bone is acting through an alternative receptor. Increasing data suggests that the IGF-1 receptor shares similar downstream events with INSR [6]. *In vitro* studies demonstrate that insulin can stimulate chondrocyte proliferation, maturation, and activity in collaboration with the local production of IGF-1 in the pancreas. Insulin exerts direct anabolic effects and promotes chondrocyte maturation *in vitro*. [7, 11]. Insulin administration *in vivo* normalizes glycemic control and reduces advanced glycation end products (AGE) production, thus it may also enhance chondrocyte survival indirectly [12, 13]. Insulin treatment on fracture healing in diabetic patients both *in vitro* and *in vivo* have shown to promote chondrocyte maturation leading to endochondral ossification [7, 11-13, 55]. Poor bone regeneration is thought to result from a decrease in insulin receptor activation which is needed to transmit signals to initiate tissue growth and repair, biomacromolecule synthesis, and glucose uptake in tissues.

Zn^{2+} is the second most common trace metal found in the body, after iron, and is the fourth most used metal in the world, after iron, aluminum, and copper [66]. Zn^{2+} has been used since the time of the ancient Egyptians, in the form of zinc oxide (ZnO), to aide in the healing of

wounds and burns [67]. Zinc chloride ($ZnCl_2$) have been shown to improve bone healing outcomes and accelerate bone healing in both non-diabetic and diabetic animal models [9]. Cellular Zn^{2+} levels are tightly regulated, and disturbances of Zn homeostasis have been associated with diabetes mellitus. Zn^{2+} dyshomeostasis, both systemically and in the pancreas, plays an intricate role in the pathology of both type 1 and type 2 DM [29]. Insulin mimetics have an insulin-like action both *in vitro* and *in vivo* experiments, but they do not build fat like insulin. It is important to discuss this pathway in detail and to highlight the effect of Zn^{2+} on these signaling events in *in vitro* and *in vivo* systems [29]. In 1980, the first report of the effectiveness of a zinc compound to stimulate rat adipocyte lipogenesis, mimicking action of insulin [22]. $ZnCl_2$ was observed to have anti-diabetic effects in streptozotocin-induced type 1 diabetic rats (STZ-rats) when $ZnCl_2$ was administered orally following the rat adipocyte experiment previously mentioned. [23, 24, 25].

Insulin-like Effects of $ZnCl_2$

Zinc compounds are used every day in various forms like an astringent, antifungal, antiseptic, skin protectant, and desensitizer for dentin. Zinc is an intracellular signaling molecule that plays important roles in growth and development, sensory function, blood clotting, reproduction, and immune system [27, 28]. Zn^{2+} enhances tyrosine phosphorylation of multiple receptor protein tyrosine kinases (R-PTKs), such as insulin receptor (IR), insulin-like growth factor type-1 receptor (IGF-1R), and epidermal growth factor (EGFR). One of the mechanisms by which thisis achieved involve an increased production of reactive oxygen species (ROS), leading to the inhibition of protein tyrosine phosphatase (PTPase) resulting in an increased tyrosine phosphorylation of R-PTK. Activated R-PTKs phosphorylate multiple downstream targets such as insulin receptor substrates (IRSs), leading to the phosphorylation and activation

of the MAPK/ERK1/2 and the phosphoinositide 3-kinase (PI3-K)/protein kinase B (PKB)/Akt (protein kinase B) signaling pathways. ERK1/2 pathways through the activation of

Figure 6: The potential mechanism for the insulin-like effects of Zn^{2+}. *Source Vardatsikos et al. (2013).*

transcription factors contribute to the increased nuclear activity, including gene transcription, cell growth, and proliferation. PI3-K/PKB/AKT pathway, through its downstream targets, such as Forkhead box protein O1 (FOXO1), glycogen synthase kinase 3-beta (GSK3-β) and mammalian target of rapamycin (mTOR) signals an increase in glucose uptake, glucose transport, glycogen synthesis, lipogenesis and inhibition of lipolysis and gluconeogenesis [9]. It also has potent antioxidant and anti-inflammatory properties [27] and has been shown to exert anti-diabetic effects in various experimental models and in a limited number of human studies [29]. Unfortunately, very little is known about the effects of zinc chloride on acceleration of fracture healing in diabetics. Previous studies of zinc chloride as an insulin mimetic do not provide a concise understanding on the mechanisms which zinc chloride uses to accelerate fracture healing in diabetics [22].

Methodology

Cell Culture

ATDC5 chondrogenic cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), and 1% L-glutamine, selenium, penicillin/streptomycin, and transferrin. ATDC5 cells were plated from a frozen stock stored in a -80ºC freezer. The frozen stock vile was warmed to room temperature, and then cells were suspended in growth media and centrifuged for five minutes at 3000rpm. The supernatant was decanted, and the cells were suspended in fresh growth media and plated as needed. Cells were checked using an electron microscope to determine confluence, the possibility of contamination, and cell death. When cells reached 100% confluence, experimental treatments were conducted as described below.

$ZnCl_2$ Treatment

ATDC5 cells were treated with increasing doses of zinc chloride ($ZnCl_2$) (0µM, 100µM, 1000µM) in triplicate over specific timepoints (2, 4, 24, and 48 hours). $ZnCl_2$-treated ATDC5 cells were harvested in Mammalian Protein Extraction Buffer (mPer) at designated timepoints (2, 4, 24, and 48 hours) for protein expression and RiboZol™ RNA Extraction Buffer for RNA expression.

Protein Isolation

Adherent $ZnCl_2$-treated ATDC5 cells were harvested with Mammalian Protein Extraction Buffer at specific timepoints while on ice. Growth media containing $ZnCl_2$ treatment was aspirated from the plates, rinsed with cold 1X HBSS, and then aspirated again to remove fluid. Mammalian Protein Extraction Buffer was added to each well and the plate was placed onto a shaker for ten minutes to coat cells evenly while on ice. Next, a cell scraper was used remove

adherent cells off the plate, assist in cell lysis, and extract proteins for western blot analysis. The lysates for each well were stored in the -20ºC until needed for further analysis.

Western Blotting

Triplicate sets of frozen protein samples in mPer buffer were thawed on ice until completely defrosted to prevent protein degradation. In separate microtubes, protein sample and 2X SDS with 2-Mercaptoethanol (2-Me) were combined in a 1:1 ratio and centrifuged at 4ºC. The protein mixture was heated in a dry bath at 70ºC for ten minutes to denature the proteins. A 4-12% NuPAGE Bis-Tris gel was run using NuPAGE MOPS Running Buffer solution. The gel was run for 50 minutes at 200V. Transfer apparatus and four pieces of filters paper were soaked in transfer buffer solution with methanol; polyvinylidene fluoride (PVDF) membrane was soaked for one minute in methanol only and rinsed in the NuPAGE transfer buffer solution. The transfer chamber was set up and sample transfer was for one hour at 30V and 100W. Following transfer, PVDF membranes were placed in SuperBlock solution (ThermoScientfic, Rockford, IL) for one hour and rocked at room temperature.

PVDF membranes were rinsed 3X in 1X Transfer Buffer Saline with 0.1% Tween (TBS-T) for five minutes then placed in primary antibody solution, stored +4ºC refrigerator, and slowly shaken overnight. The primary antibody solution consisted of 10µM GAPDH, 10µM Akt (pan) or phospho-Akt (pan), 0.1% SuperBlock, and 1X TBS-T. After overnight incubation, PVDF membranes were rinsed 3X in 1X TBS-T for five minutes then placed in secondary antibody for one hour and shaken at room temperature. The secondary antibody solution consisted of 0.2µM anti-goat IgG-conjugated antibody, 0.2µM anti-mouse IgG-conjugated antibody, TBS-T, and 0.1% SuperBlock. The membranes were rinsed twice in 1X TBS-T and once in 1X TBS to remove any remaining secondary antibody then placed in Pierce® Enhanced Chemiluminescence

(ECL) Western Blotting Substrate to view under UV light using the FluorChem E System (ProteinSimple, San Jose, CA).

RNA Isolation

To prepare for RNA isolation of confluent adherent cells, all equipment was sterilized with 70% ethanol and confined to a specific location within the laboratory to minimize contamination and RNA degradation by RNases. ATDC5 cells were treated with $ZnCl_2$ at 0µ, 100µM, 1000µM dosages and seeded (triplicate) in 6-well plates with similar method used for protein isolation. Post-treatment, media was aspirated and RiboZol™ RNA Extraction Reagent was used to isolate RNA. ATDC5 cells were lysed by passing them through the tip of the pipette several times to disrupt the cell wall and release RNA. Next, lysed ATDC5 cells were transferred into an RNase-free microtubes and incubated at room temperature for five to ten minutes. RiboZol™ solution was mixed with 100µL of chloroform in a tightly secured microtubes and shaken vigorously for fifteen seconds to thoroughly mix the samples; this solution was incubated at room temperature for three minutes to allow phases to begin to separate. The samples were centrifuged at 12,000rpm for fifteen minutes at +4ºC to separate the mixture into three phases: clear aqueous phase (RNA), white interphase (DNA), and pink phenol-chloroform phase (protein).

Up to 80% of the clear aqueous phase exclusively containing RNA was carefully removed by pipette, placed in a fresh RNase-free microtube, and a volume of isopropanol was added to initialize homogenization. The samples were incubated for ten minutes at room temperature then centrifuged at 12,000rpm for ten minutes at +4ºC. Supernatant was removed without disrupting the precipitate RNA pellet that was located at the bottom of the RNase-free microcentrifuge tube. The pellet was washed once with a volume of 75% ethanol that was

prepared with DEPC-treated RNase-free water, vortexed and centrifuged at 7,500rpm for five minutes at +4ºC. The ethanol wash was carefully removed without dislodging the RNA pellet. The pellet was briefly air-dried for five to ten minutes at room temperature until most of the remaining alcohol evaporated from the microcentrifuge tube. The RNA pellet was re-dissolved into RNase-free water and passed through a pipette tip several times until completely dissolved. RNA yield and purity were determined by absorbance at A260 with a BioDrop DUO (UK, England) immediately after isolation process. RNA purity was determined by the ratio of absorbance (A260/280). Purity and concentration of RNA were confirmed via gel electrophoresis described below.

RNA Gel

RNA samples were prepped for a 1% agarose gel to verify that RNA was intact after isolation process. 1% agarose gel was made with 1X 3-(N-Morpholino)propanesulfonic Acid (MOPS) buffer. Approximately 20-25mL of agarose solution was poured into the gel electrophoresis apparatus then allowed to solidify for 20-40 minutes. 1X MOPS buffer was poured over the gel until submerged and acted as running buffer during the process. The samples were prepped in separate RNase-free microcentrifuge tubes and included 1µg of RNA sample, 1X Sucrose Gel Loading Dye, and RNase-free water. All prepped samples were vortexed, centrifuged, and loaded into the agarose gel. The gel apparatus was set at 90V and ran for one hour; the RNA gel was placed on a rocker at room temperature for fifteen minutes in RNase-free water with GreenGlo™ Safe DNA Dye. The results of the RNA gel were visualized under UV light and RNA samples were viewed; RNA samples that were confirmed to be intact were used for reverse transcription (RT). The original RNA samples were stored at -80ºC to prevent degradation.

Reverse Transcription (RT)/Polymerase Chain Reaction (PCR)

RNA samples were removed from -80ºC storage and thawed on ice; two master mixes were prepared for reverse transcription (RT) and placed on ice until aliquoted into RNA samples. One microgram of each RNA sample was combined with 5mM dNTPs and 5mM oligo dTs and brought up to final volume of 16µL with RNAse free water. The RNA samples were vortexed and centrifuged for thirty seconds at 12,000rpm. The samples were inserted into a dry bath set at 65ºC for three minutes for denaturation then immediately returned to ice to reduce degradation. These cooled RNA samples were combined with 2µL of 10X Standard RT (Taq) Buffer, 1µL of RNAOUT, and 1µL of reverse transcriptase enzyme. Combined RNA reactions were vortexed, and centrifuged again for thirty seconds at 12,000rpm. RNA reactions were incubated in a water bath set at 42ºC for one hour. After incubation, microcentrifuge tubes were placed in a dry bath at 95ºC for ten minutes. Post-denaturation, RT samples were cooled at room temperature to promote annealing. RT samples were prepared for polymerase chain reaction (PCR) to confirm the presence of complimentary DNA (cDNA).

Each PCR reaction contained 2.5µL of cDNA, 2.5µL 10X Standard RT (Taq) Buffer, 2.5µL 5mM dNTPs, 1.5µL 10mM primer mix, and 0.125µL Taq polymerase. The samples were vortexed then placed in the PCR machine and the reaction proceeded for two hours and fifteen minutes. DNA gel was performed to confirm RT/PCR process was successful. Table 1 is the thermocycling conditions used for routine PCR:

Table 1: Thermocycling conditions for a routine PCR.

STEP	***TEMP (ºC)***	***TIME***
Initial Denaturation	95ºC	30 seconds
40 Cycles	95ºC	15-30 seconds
	45-68ºC	15-60 seconds
	68ºC	1 minute/kb
Final Extension	68ºC	5 minutes
Hold	4-10ºC	

DNA Gel Electrophoresis

DNA gel electrophoresis was setup similarly as a RNA gel, except 1X TBE Buffer is used instead of 1X MOPS Buffer with a 2% agarose gel. cDNA samples were combined with 1X Sucrose Loading Dye and ran for one hour at 90V. The DNA gel was submerged in dH_2O with GreenGlo™ Safe DNA Dye and rocked for fifteen minutes. DNA gel was viewed under UV light to determine if RNA was successfully converted into cDNA during PCR process.

Quantitative PCR (qPCR)/Gene Expression

Prior to qPCR, cDNA samples were diluted 1:10 to increase number of reactions that could be performed using a 96-well qPCR plate; 2.5µL of cDNA was added prior to distribution of the master mix into each well. The following solutions were thawed on ice and combined into a sterile microcentrifuge tube for each reaction: 12µL of 2X Absolute Blue SYBR, 1.75µL each of 1µM forward and reverse primer per sample, 6.5µL per sample RNase-free dH_2O. The primers used for qPCR and gene expression were glyceraldehyde-3-phosphate dehydrogenase (GAPDH – housekeeping gene) for normalization, collagen type II alpha 1 chain (COL2A1), collagen type X alpha1 chain (COL10A1), Runx2, mTOR, ALP, and β-actin (Table 4). Total volume of the master mix solution was 22.5µL per well. The total volume of each well for this procedure was

25µL (22.5µL of master mix plus 2.5µL of cDNA). Tables 2 and 3 are the qPCR thermal cycling program and melt curve program:

Table 2: qPCR Thermal Cycling Program

	Temperature (ºC)	***Time***	***Number of cycles***
Enzyme Activation	95ºC	15 min.	1
Denaturation	95ºC	15 sec.	40
Annealing	50-60ºC	30 sec.	40
Extension	72ºC	30 sec.	40

Table 3: Melt Curve Program

	Temperature (ºC)	***Time***	***Number of cycles***
Denaturation	95ºC	30 sec.	1
Starting Temp.	60ºC	30 sec.	1
Melting Temp.	60ºC	10 sec.	80

Table 4: Primer sequences for detection of COL2A1, COL10A1, β-actin, RUNX2, and mTOR were based on the coding sequences of the respective mRNAs.

Oligo Name	**Sequence (5' to 3')**
gapdh_F	ACCACAGTCCATGCCATCAC
gapdh_R	TCCACCACCCTGTTGCTGTA
COL2A1_F	TGGGTGTTCTATTTATTTATTGTCTTCCT
COL2A1_R	GCGTTGGACTCACACCAGTTAGT
COL10A1_F	CATGCCTGATGGCTTCATAAA
COL10A1_R	GCGTTGGACTCACACCAGTTAGT
mTOR_F	CCATCCAATCTGATGCTGGA
mTOR_R	GGTGTGGCATGTGGTTCTGT
RUNX2_F	GACGAGGCAAGAGTTTCACC
RUNX2_R	GGACCGTCCACTGTCACTTT
ALP_F	CCGATGGCACACCTGCTT
ALP_R	GGAGGCATACGCCATCACAT
B-actin_F	GACAGGATGCAGAAGGAGATTACTG
B-actin_R	CCACCGATCCACACAGAGTACTT

The C_T values for each gene (Table 4) were normalized with GAPDH C_T values. Gene expression was calculated using the 2-ΔΔCt method.

Alizarin Red Staining

Briefly, calcium containing cells were determined by Alizarin Red Staining (ARS). ATDC5 cells were treated under four different conditions in 12-well plates and harvested at timepoints: 2, 4, 7, 10, 14, 17, 21, and 28 days. The cells were treated in triplicate with 1ug/ml of insulin, 10μM of $ZnCl_2$, 100μM of $ZnCl_2$ and 100μM of vanadium. The 12-well plates were washed with HBSS then fixed with 10% (v/v) formaldehyde at room temperature for 15 minutes. Next, plates were washed twice with HBSS and 40mM Alizarin Red Staining (ARS) solution was pipetted into each well. The ARS stained plates were placed very carefully on a rocker at room temperature for 20 minutes. ARS solution was aspirated from ATDC5 cell plates then washed four times with HBSS while shaking for five minutes. To facilitate the removal of any remaining liquid, the plate was angled for two minutes and re-aspirated. For quantification of staining, 10% (v/v) acetic acid was added to each well and incubated at room temperature for 30 minutes on a rocker. Using a cell scraper, the monolayer was scraped from the plate and transferred with 10% (v/v) acetic acid to a clean centrifuge tube. The samples were vortexed briefly for 30 seconds then warmed for 10 minutes in a dry bath at 85ºC. Afterwards, the samples were placed on ice for five minutes then centrifuged at 13,000rpm for 15 minutes. 500μL of supernatant was transferred into a new centrifuge tube and 200μL of 10% (v/v) ammonium hydroxide was added to the supernatant. Supernatant was analyzed in aliquots of 150μL in triplicate in a 96-well plate and read at 405nm.

Glacial Blue Staining

ATDC5 cell proteoglycans were treated under four different conditions in 12-well plates and harvested at timepoints: 2, 4, 7, 10, 14, 17, 21, and 28 days. The cells were treated in triplicate with 1ug/ml of insulin, 10μM of $ZnCl_2$, 100μM of $ZnCl_2$ and 100μM of vanadium. The 12-well plates were washed twice with HBSS then fixed with 100% cold methanol for five minutes. Next, plates were stained with 0.1% Alcian Blue in 0.1M HCl and rocked overnight. The ATDC5 cell monolayers were washed three times with HBSS. Proteoglycans were extracted with 6M guanidine-HCl overnight. Proteoglycan concentration was read at 595nm.

Results

Treatment with Zinc Chloride Activates Phospho-Akt and Akt Protein Expression

Zinc chloride ($ZnCl_2$) has been shown in several studies to induce chondrogenesis and promote chondrocyte maturation [22, 35-38]. To elucidate possible mechanisms by which $ZnCl_2$ induced chondrogenesis, phospho-Akt and Akt expression levels were measured at 2, 4, 24, and 48 hours post-treatment in the ATDC5 chondrogenic cell line. Figure 8 shows that $ZnCl_2$ treatment upregulated phospho-Akt/Akt expression greater than the control, 0μM $ZnCl_2$ group. The 1000μM $ZnCl_2$ dose showed significantly more phospho-Akt expression compared to both 0μM $ZnCl_2$ and 100μM $ZnCl_2$ dosage groups at 2, 4, and 24 hours. The 1000μM $ZnCl_2$ dose increased phospho-Akt expression by 19-fold, at 4 and 24 hours post-treatment ($P<0.025$). Phospho-Akt/Akt expression treated with 0μM $ZnCl_2$ did not activate phospho-Akt/Akt

expression. ATDC5 cells show a 4, 5, 15-fold increase when treated with 1000μM $ZnCl_2$ at 2, 4, and 24 hours post-treatment represented by Figure 7.

Figure 7: Phospho-Akt Protein Expression of Zinc Chloride ($ZnCl_2$) Treated ATDC5 Cells.

Figure 8: Phospho-Akt and Akt protein expression in $ZnCl_2$ treated ATDC5 cells. Cells were treated with denoted concentrations of ZnCl2. A) 4 hours; B) 48 hours.

Figure 8 is an immunoblot of phospho-Akt and Akt expression at 4 and 48 hours post-treatment. At 4 hours post-treatment, both phospho-Akt and Akt are expressed in all $ZnCl_2$ doses. The 1000μM $ZnCl_2$ dose had significant phospho-Akt antibody expression at 4 hours compared to the 0μM and 100μM $ZnCl_2$ doses. At 48 hours post-treatment there was little to no phospho-Akt expression in any treatment group, but Akt protein was expressed in all $ZnCl_2$ doses.

Treatment with Zinc Chloride Results in Upregulation of Chondrocyte Gene Expression Markers

Collagen markers were shown to have specific binding with cDNA generated from PCR. Figure 9 shows that $ZnCl_2$ treated ATDC5 cells expressed specific gene markers at 2, 4, 24, and 48 hours post-treatment when normalized with housekeeping gene, GAPDH. Collagen type II alpha chain is expressed in early chondrocyte maturation while collagen type X alpha chain is expressed by hypertrophic chondrocytes prior to apoptosis.

Figure 9: Gene expression of ZnCl2 treated ATDC5 cells over time.

The 100μM $ZnCl_2$ dose caused a 2.9- ($P=0.011$), 6.1- ($P<0.001$), and a 1,6-fold ($P<0.001$) increase in *col2a1* mRNA levels at 2, 24, and 48 hours (Figure 9A). The 1,000μM $ZnCl_2$ dose also increased *col2a1* mRNA levels by 3.9- ($P=0.003$), and 7.6-fold ($P<0.001$) at hours 24 and 48 hours. At 48 hours, the 100μM $ZnCl_2$ treated ATDC5 cultures had 0.25-fold less ($P=0.009$) collagen type X alpha chain 1 (*col10a1*) mRNA as compared to 0μM $ZnCl_2$ (Figure 9B). The 1,000μM dose of $ZnCl_2$ significantly reduced *col10a1* expression as compared to controls at 4

(P=0.018) and 48 (P<0.001) hours after treatment. These data suggest that the 100μM dose of $ZnCl_2$ stimulates chondrocyte differentiation and that the 1,000μM dose may inhibit this process. Other gene markers were quantified to determine the role of $ZnCl_2$ in the promotion of chondrogenesis. There were no significant differences found in Runx2 expression when compared between treatment groups over time (Figure 9C). The 100μM $ZnCl_2$ dose reduced mTOR gene expression at 24 hours (P=0.011) as compared to the control groups but no other significant differences were detected. The 1000μM $ZnCl_2$ dose significantly reduce mTOR expression at 48 hours (P=0.005) as compared to the control group (Figure 9D).

Treatment with Zinc Chloride Upregulates Proteoglycan Synthesis

Figure 10: Quantification of Proteoglycans after $ZnCl_2$ treatment over time.

Proteoglycan synthesis was measured with Alcian Blue staining on insulin-treated and 100μM and 1000μM $ZnCl_2$ dose treated ATDC5 cell cultures. $ZnCl_2$ treatment was shown to promote proteoglycan synthesis in ATDC5 cells and statistical differences were found after two-way ANOVA analysis (Table 5). The data showed that proteoglycan synthesis was highest

between days 4 and 14 in both control and 100μM $ZnCl_2$ treated ATDC5 cells (Figure 10, Table 5). On day 4, controls had produced significantly more proteoglycans than either $ZnCl_2$ treatment group. After two weeks (day 14), 100μM $ZnCl_2$ treated cells had 1.5-fold more proteoglycans when compared to insulin-dependent media (IDM) controls. Also, the data analysis shows the 1000μM $ZnCl_2$ dose had a significant decrease in proteoglycan concentration between days 4 and 14 and demonstrated high levels of cell death after treatment.

Table 5: Two-Way Analysis of Variance: Effect on Treatment on Proteoglycan with time points.

	Comparison of Treatment Groups at Each Time Point (Holm-Sidak Test P Values)		
	100uM $ZnCl_2$ vs Control	1000uM $ZnCl_2$ vs Control	100uM $ZnCl_2$ vs 1000uM $ZnCl_2$
2	0.989	0.983	0.904
4	0.001	<0.001	<0.001
7	0.838	<0.001	<0.001
10	<0.001	<0.001	<0.001
14	0.532	<0.001	<0.001
21	0.204	0.402	0.062

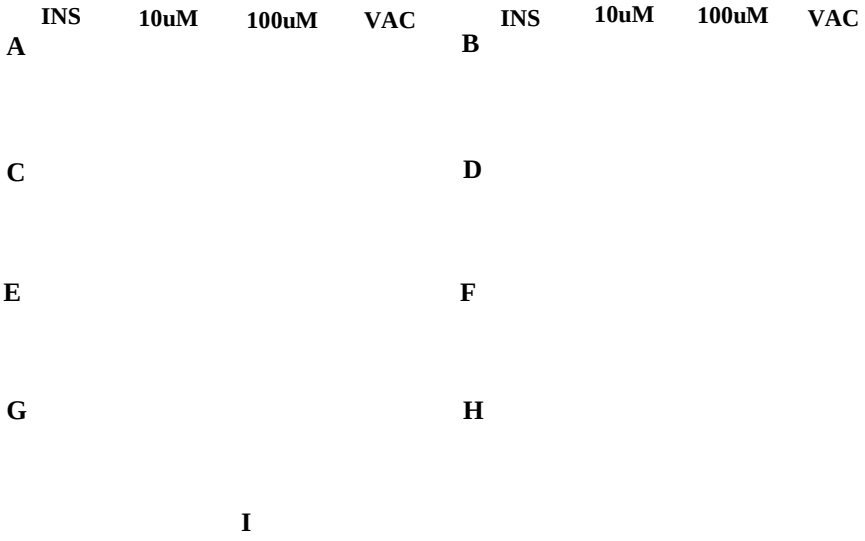
Treatment with Zinc Chloride Promotes Calcium Deposition

In comparison to proteoglycan synthesis, calcium deposition was measured during chondrocyte differentiation with Alizarin Red staining (ARS) on $ZnCl_2$ treated ATDC5 cells. ATDC5 cells were treated every two days under three conditions: IDM (control), 10μM and 100μM $ZnCl_2$, and VCl_2 after reaching confluence. Cells were harvested on days 2, 4, 7, 10, 14, 17, 21, 24, and 28 days post-treatment. Based on statistical data analyzed with two-way analysis of variance test using time and treatment as factors, calcium deposition was shown to be similar for 100μM $ZnCl_2$ dose and IDM control samples between days 2-17 (Figure 11 and 12). At days 21, 24, and 28, the 100μM $ZnCl_2$ dose increased calcium deposition when compared to IDM controls. However, there were no significant differences in calcium deposition between ATDC5

cells treated with 10μM $ZnCl_2$ and IDM controls at any time point (data not shown). After day 7, the 1000μM $ZnCl_2$ dose caused significant cell death in ATDC5 cells and was not evaluated in ARS assay.

Figure 11: Quantification of Calcium Deposition of $ZnCl_2$ Treatment.

Figure 12: ARS stain assay on $ZnCl_2$ treated ATDC5 cells. A) 2 days; B) 4 days; C) 7 days; D) 10 days; E) 14 days; F) 17 days; G) 21 days; H) 24 days; I) 28 days *Source Burgess (2017)*



Discussion

Zinc is an essential micronutrient required for many cellular processes, especially for the normal development and function of the immune system [68]. Zinc is an essential trace element to the function of metabolism [69]. Zinc deficiency results in a retardation of bone growth, development, and maintenance of bone health [70,71]. $Zinc^{2+}$ acts to increase bone formation and mineralization, decrease bone resorption and stimulate alkaline phosphatase (ALP) activity, in both calvaral organ cultures and osteoblast cell cultures [72-75]. Studies have demonstrated that zinc has an anabolic stimulatory effect on bone formation and mineralization *in vivo* and *vitro*. For example, Yamaguchi and Takahashi demonstrated that administration of zinc sulfate for 3 days produced dose dependent increases in the contents of zinc, deoxyribonucleic acid (DNA), collagen and calcium, and the activity of ALP in the femoral diaphysis (cortical bone) of weanling rats [76]. Zinc activation of ALP in bone tissues may first cause activation of ALP and stimulation of collagen synthesis in osteoblasts, which are involved in bone mineralization and calcification [77].

Fracture healing studies have demonstrated that insulin mimetics like zinc chloride ($ZnCl_2$) can enhance bone healing and stimulate bone growth via production of TGF-β1 and IGF-1 in bone tissues of newborn rats [78]. The mechanism by which insulin mimetic, $ZnCl_2$, improves bone healing is poorly understood. The current data suggests that this insulin mimetics promote and enhance chondrogenesis [79], osteoblastogenesis [80], and prevents osteoblast proliferation [81] by acting similar to insulin. Fukumoto et al. demonstrated *in vitro* that IGF-1 significantly increased chondrogenesis in dose-dependent manner when administered continuously throughout the culture period of periosteal explants from the medial proximal tibia of 2-month old rabbits. IGF-1 and TGF-β1 for the first 2 days, enhanced overall total cartilage

growth. Immunohistochemistry for proliferating cell nuclear antigen (PCNA) demonstrated that combining IGF-1 with TGF-β1 gave the strongest proliferative stimulus early during chondrogenesis [82]. Other data shows that *in vivo* insulin acts as an anabolic agent in bone healing and regeneration using Diabetes Mellitus Type I (T1DM) rat models because impaired insulin signaling in bone-forming cells results in a secondary and local insulin-like growth factor 1 (IGF-1) deficiency [83]. Zinc chloride's role on chondrocytes has not been thoroughly evaluated. We hypothesized that zinc chloride could enhance chondrocyte maturation and differentiation like insulin and stimulate transcriptional expression of chondrogenic markers like collagen type II alpha 1 chain (COL2A1) and collagen type X alpha 1 chain (COL10A1).

Our data shows that $ZnCl_2$ treatment can induce chondrocyte differentiation in the absence of insulin as measured by both proteoglycan quantification and calcium deposition. Our data coincides with Yamaguchi et al. who demonstrated *in vitro* that zinc could stimulate bone collagen production and increase bone calcium content in calvaria of weaning rats [73]. Our data also shows that $ZnCl_2$ induces chondrocyte differentiation by transcriptional upregulation of chondrogenic markers of COL2A1 and COL10A1. These chondrogenic markers are expressed as hallmarks of chondrogenesis and endochondral ossification. $ZnCl_2$ treatment *in vivo* demonstrated mechanical strength, including increased torque to failure, torsional rigidity, shear modulus, and shear stress [85]. In this study [85], fracture callus showed an increased percent cartilage and mineralized tissue at day 7 in $ZnCl_2$ treated group. Cell proliferation was increased by local $ZnCl_2$ in both subperiosteal and gap callus regions. VEGF and IGF-1 levels within the fracture callus were increased by local $ZnCl_2$ treatment. This study demonstrates the potential role of local $ZnCl_2$ as an adjunct for fracture healing [85]. This suggests that $ZnCl_2$ treatment contributes to growth factor expression, which stimulates cell proliferation, cell differentiation,

and enhances endochondral ossification. Our data suggests that $ZnCl_2$ upregulation of gene markers *col2a1* and *col10a1* are dose-dependent and expressed in during chondrocyte maturation as measured by gene expression quantification.

Zinc chloride has been demonstrated to possibly function through similar methods as insulin, but the mechanism by which $ZnCl_2$ induces chondrogenesis is still uncertain. Chondrogenesis has a multitude of signaling regulators and mechanisms regulate chondrocyte maturation and cartilage production. These include various signaling pathways, including fibroblast growth factor (FGF), transforming growth factor β (TGF-β)/bone morphogenetic proteins (BMPs), Wnt/β-cats in, Hedgehog, Notch, hypoxia, and angiogenic signaling pathways [86]. Our data demonstrated that $ZnCl_2$ does not induce PI3K-Akt pathway to stimulate Runx2 transcriptional expression within the first 48 hours of treatment. Runx2 expression was compared between $ZnCl_2$ treatment groups over time, no significant differences were found. Runx2 and Runx3 are essential for chondrocyte maturation and regulation of limb growth, and these effects are carried out through RunX-induced expression of Indian hedgehog (Ihh). Ihh promotes chondrocyte differentiation via expansion of Sox9 expression [86]. Our data shows that $ZnCl_2$ treatment had no effect on Runx2 expression. This suggests Runx2 is expressed through another pathway. Future experiments could extrapolate other pathways in which $ZnCl_2$ induce chondrogenesis. In addition, our gene expression show that $ZnCl_2$ reduces transcriptions expression of mammalian Target of Rapamycin (mTOR), which is an inhibitor of Insulin Receptor Substrate-1 (IRS-1) [87]. The serine/threonine kinase, mTOR, is a protein kinase of the PI3K/Akt signaling pathway [89]. Phornphutkul et al. demonstrated that in the presence of rapamycin, mTOR was still expressed in late gestation fetal rats [87]. Rapamycin had a significant effect on early hypertrophic chondrocytes and minimal effect on chondrocyte

proliferation. Effect of rapamycin was measured with a phosphorylated-S6 stain [87]. Phosphorylated-S6 is a ribosomal protein and marker for activity of mTOR. This demonstrates that mTOR inhibitor, rapamycin, does not significantly affect chondrocyte proliferation. Our data suggests that with reduced mTOR expression, it is possible that IRS-1 can upregulate expression of various downstream targets of PI3K/Akt signaling pathway. Since mTOR is an Insulin Receptor Substrate-1 (IRS-1) inhibitor, our data suggests IRS-1 may contribute in $ZnCl_2$ induced chondrogenesis. Current research suggests that FGF and insulin have a synergistic effect to induce chondrogenesis and work long-term collaboratively to produce fully functional cartilage [88]. As previously mentioned, chondrogenesis can be induced by a plethora of signaling pathways. Therefore, in the future we plan to investigate the mechanism by which $ZnCl_2$ affects chondrogenesis utilizing next generation sequencing (NGS).

Overall, we demonstrate that zinc chloride ($ZnCl_2$) can induce chondrogenesis and upregulate expression of downstream targets of both insulin and PI3K/Akt signaling pathway. We demonstrated that zinc chloride treatment on ATDC5 chondrogenic cell line promotes chondrocyte differentiation as seen by transcription upregulation of chondrogenic hallmarks, COL2A1 and COL10A1. 100μM $ZnCl_2$ treatment group was the most abundant of proteoglycan content and calcium deposition when compared with IDM controls. This demonstrates that $ZnCl_2$ was capable of inducing chondrocyte differentiation *in vitro*.

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